GLUTATHIONE DERIVATIVES AS INHIBITORS OF GLUTAREDOXIN AND RIBONUCLEOTIDE REDUCTASE FROM *ESCHERICHIA COLI*

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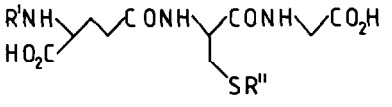
1. Introduction

Ribonucleotide reductase requires a dithiol as hydrogen donor for reduction of ribonucleotides to the corresponding deoxyribonucleotides [1,2]. Originally, thioredoxin, a small dithiol protein, was found to be the hydrogen donor in the reaction [1]. The oxidized thioredoxin (thioredoxin-S_2) is reduced by the specific enzyme NADPH-thioredoxin reductase. In [3], another system was found in an *Escherichia coli* mutant lacking detectable thioredoxin. The monothiol, glutathione, is hydrogen donor in the presence of glutaredoxin, a novel small protein that thus couples the oxidation of glutathione to the reduction of a ribonucleotide [3]. To study the mechanism of action of glutaredoxin we have used the glutathione analogues **1**–**5** shown below in scheme 1. Our results provide evidence for a glutathione-binding site on ribonucleotide reductase.



1 ; $R' = C_6H_5CH_2OCO-$, $R'' = H$

2 ; $R' = H$, $R'' = -CH_2C_6H_4pBr$

3 ; $R' = H$, $R'' = -CH_2COC_6H_5$

4 ; $R' = H$, $R'' = -CH_2COC_6H_4pN_3$

5 ; $R' = R'' = -COOCH_2C_6H_5$

Scheme 1

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2. Materials and methods

NADPH, CDP, ATP and GSH were obtained from Sigma. [^{3}H]CDP was from Amersham. Glutaredoxin was prepared from *E. coli* C10-17 by an immunoadsorbent technique (in preparation). Ribonucleotide reductase from *E. coli* was a mixture of proteins B1 and B2 [1] and was of 95% purity; it was a generous gift from Dr B.-M. Sjöberg, Department of Biochemistry, Karolinska Institute. Glutathione reductase (highly purified from yeast) was from Sigma. Bovine serum albumin was from British Drug Houses. Thioredoxin [4] and thioredoxin reductase [5] were preparations from *E. coli* B. The glutathione analogues **2**–**4** were from previous studies [6]. Derivatives **1** and **5** were prepared by a modification of the procedure in [7] and were found to be pure by thin-layer chromatography and elemental analysis. However, **1** was found to oxidize easily and the thiol content had dropped to 75% at the time of the experiment. Stock solutions of derivatives **1**–**5** were prepared in dimethyl sulphoxide. This solvent alone was shown to have only small effects in the assay of the activity of ribonucleotide reductase; <5% inhibition at 8% DMSO (v/v). The activity of ribonucleotide reductase in the presence of inhibitors of the glutaredoxin system was assayed by determining the conversion of [^{3}H]CDP to d[^{3}H]CDP as in [8]. Relevant concentrations were: *E. coli* ribonucleotide reductase (subunits B1 + B2) 0.3 μM; NADPH 1 mM; [^{3}H]CDP 0.5 mM; glutaredoxin 0.5 μM, glutathione reductase 8 μg/ml and GSH and GSH-analogue concentrations were as indicated. In assays of ribonucleotide reductase with the thioredoxin system, GSH and glutathione reductase were exchanged for thioredoxin 4 μM, and excess thiore-

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doxin reductase (15 μg/ml). Incubations were for 20 min at 37°C in 120 μl final vol. Assays of thioredoxin in the insulin disulfide reduction system were performed as in [9] by following the oxidation of NADPH spectrophotometrically.

3. Results

In the glutaredoxin assay system, derivatives **1** and **2** were found to be linearly competitive with glutathione by both Lineweaver-Burk ($1/V_0$ *vs* $1/S_0$) and Dixon ($1/V_0$ *vs* I_0) criteria, the results for **2** being depicted in fig.1. The K_i values for **1** and **2** were found to be 0.38 ± 0.05 mM and 0.80 ± 0.04 mM, respectively (see table 1) by calculation from the mean intersections on the $1/S_0$ axis of the appropriate Lineweaver-Burk plots. Compound **3** gave a linear Lineweaver-Burk plot with the intersection on the $1/V_0$ axis constant for various inhibitor levels. However, the Dixon replot of these data may indicate a slight curvature at lower substrate concentrations (see fig.2). If a linear competitive model is assumed, the K_i for **3** in the glutaredoxin system is 1.2 ± 0.2 mM. Well-defined, non-linear inhibition behavior (Dixon plot) was found for the 4-azido analogue **4**, and for **5**,

Table 1
Inhibition of glutaredoxin- and thioredoxin-dependent ribonucleotide reductase by glutathione derivatives

Derivative	K_i (mM) in glutaredoxin system	Concentration for 50% inhibition (I_{50}), mM of thioredoxin system
1[a]	0.38 ± 0.05	3.5
2	0.80 ± 0.04	[b]
3	1.2 ± 0.2	1.8
5	–[c]	3.0

[a] Concentrations of **1** were calculated by weight; the free SH titer was found to be 75% of theoretical
[b] No inhibition detected
[c] Not calculated

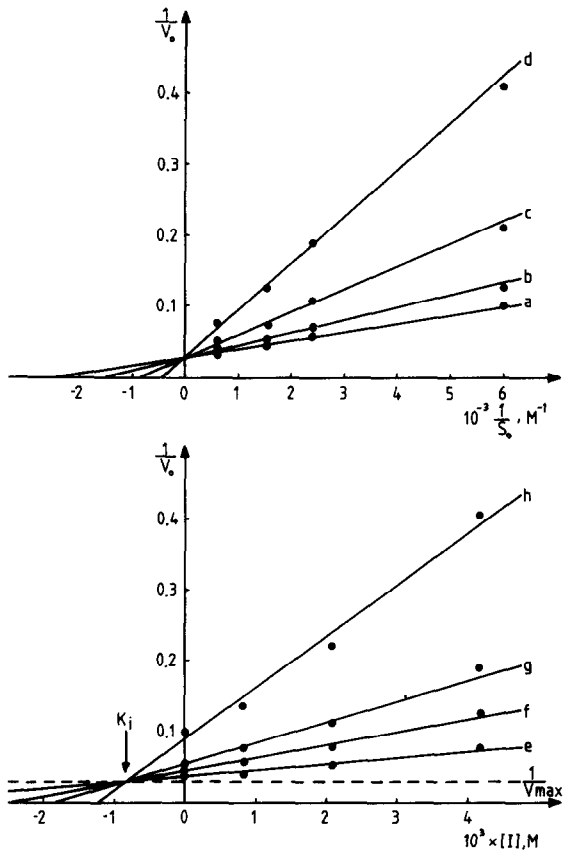

Fig.1. Lineweaver-Burk (top) and Dixon (bottom) plots of the inhibition of glutaredoxin-dependent ribonucleotide reductase by *S*-(*p*-bromobenzyl)glutathione (**2**). Velocity units are nmol dCDP formed/20 min. The points are experimental and the lines derived by regression analysis of the data assuming linear, competitive inhibition with K_i = 0.80 ± 0.04 mM. The inhibitor concentrations (upper figure) were: (a) 0 mM; (b) 0.83 mM; (c) 2.1 mM; (d) 4.2 mM. The GSH concentrations (lower figure) used were: (e) 1.7 mM; (f) 0.83 mM; (g) 0.42 mM; (h) 0.17 mM.

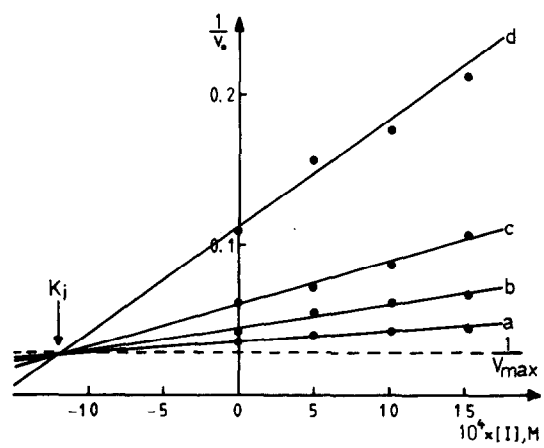

Fig.2. Dixon plot for *S*-(phenacyl)-glutathione inhibition of glutaredoxin-dependent ribonucleotide reductase. Velocity units are nmol dCDP formed/20 min. The points are experimental and the lines are theoretical for linear competitive inhibition with K_i = 1.2 ± 0.2 mM (obtained from the Lineweaver-Burk intersects). The GSH concentrations used were: (a) 1.7 mM; (b) 0.83 mM; (c) 0.42 mM; (d) 0.17 mM.

blocked on both N and S sites, but these complex inhibitors were not investigated further.

All of the compounds used with the exception of 2 were inhibitory in the thioredoxin-dependent assay of ribonucleotide reductase. The concentrations required for 50% inhibition are given in table 1. Inhibition types were more complex than in the glutaredoxin-based system; e.g., that for 3 was non-competitive with a K_i of 2 mM. In view of the relatively weak inhibitions of the thioredoxin assay system and the complex inhibition kinetics observed, this aspect was not studied in detail. None of the compounds shown in table 1 had any detectable effect on the insulin-reducing activity of thioredoxin. S-Blocked and N,S-diblocked glutathione derivatives showed little, if any, inhibition of yeast glutathione reductase; in any case this enzyme is used in excess in the assay.

4. Discussion

From the results obtained with thioredoxin-dependent ribonucleotide reductase it is clear that GSH analogues can inhibit, albeit weakly, this activity (table 1). The fact that these compounds do not inhibit, to any detectable level, the insulin disulfide reducing ability of thioredoxin argues strongly that there is a GSH-analogue binding site on ribonucleotide reductase. This in turn implies a GSH-site on that enzyme. In this context it should be pointed out that excess GSH is not inhibiting ribonucleotide reductase in the presence of thioredoxin. It thus seems likely that the binding of the GSH analogues to a GSH site on ribonucleotide reductase interacts with the function of the active site.

Interpretation of the results for glutaredoxin-dependent ribonucleotide reductase is more complex. There is undoubtedly a GSH-site in this system which can be blocked by the derivatives tested. The observation of linear competitive inhibition by 1 with a K_i fairly close to the K_m (0.4 mM) for GSH itself [10] argues that for 1 this site is the active site. Indeed, there must be little steric constraint around the thiol group of GSH in the active site in view of the large *p*-bromobenzene group. This statement has to be qualified because of the difference in inhibitory behavior between the *S*-(phenacyl)-(3)- and *S*-(*p*-azidophenacyl)-(4)-derivatives. It is not possible on the current evidence to decide the location of this GSH-site. It may be on the glutaredoxin and/or ribonucleotide reductase. It is clear from table 1, comparing results for 1 and 3, that these inhibitions involve specific interactions and are not the result of random binding to ribonucleotide reductase. Thus, in the glutaredoxin system 1 is a significantly better inhibitor than 3, but the reverse is the case in the thioredoxin system. More strikingly, 2 does not inhibit the thioredoxin system, detectably, but is a reasonably strong inhibitor for the glutaredoxin system.

GSH-derivatives inhibit, in a specific manner, ribonucleotide reductase from *E. coli* as assayed by both glutaredoxin and thioredoxin-dependent systems. While it is not yet possible to assign the inhibition site for the glutaredoxin system, there appears to be clear evidence of a GSH-site on ribonucleotide reductase when assayed in the presence of the thioredoxin system.

Acknowledgements

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